AD_____

Award Number: DAMD17-01-1-0346

TITLE: Antiangiogenic Action of Chemically Modified Tetracyclines in Breast Cancer

PRINCIPAL INVESTIGATOR: Sanford R. Simon, Ph.D.

CONTRACTING ORGANIZATION: State University of New York
at Stony Brook
Stony Brook, New York 11794-3362

REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (15 May 2001 - 14 May 2002)

4. TITLE AND SUBTITLE

Antiangiogenic Action of Chemically Modified Tetracyclines in Breast Cancer

5. FUNDING NUNUMBER
DAMD17-01-1-0346

6. AUTHOR(S)

Sanford R. Simon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

State University of New York at Stony Brook
Stony Brook, New York 11794-3362
email - ssimon@notes.cc.sunysb.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The long term objective of this project is to evaluate the capacity of a group of nonantimicrobial chemically modified tetracyclines (CMTs) to downregulate the angiogenic response which maintains breast tumor growth and proliferation. In this first year we determined levels of the angiogenic factor VEGF released by two breast tumor cell lines, MCF-7 and MDA-MB-231 and demonstrated dose-dependent inhibition of VEGF release from both cell lines cultured for 24 hours with CMT-3 or CMT-308. CMT-308 was the more potent inhibitor of the two CMTs at all doses. Neither CMT was cytotoxic to either cell line at doses which can be achieved in patients. Addition of the growth factor TGF-β to either cell line resulted in factor-dose-dependent increases in levels of released VEGF, but the augmented VEGF levels could be diminished somewhat by CMT-3 and more significantly by CMT-308. VEGF levels released by either cell line were unaffected by IGF-1. The human monocytoid line Mono Mac 6 also released VEGF in the absence of added stimuli; VEGF levels from Mono Mac 6 were not affected by TGF-β or IGF-1, but were diminished somewhat in the presence of CMT-3 and markedly (to virtually undetectable levels) in the presence of CMT-308. CMT-3 and CMT-308 were not cytotoxic to confluent human umbilical vein endothelial cells at doses which inhibited VEGF release from the breast tumor cell lines and Mono Mac 6 cells. These results suggest that CMTs, especially CMT-308, may be of use as antiangiogenic agents in management of breast cancer.

14. SUBJECT TERMS
breast cancer, angiogenesis, tetracyclines, macrophages, breast tumor cell lines, endothelial cells, VEGF

15. NUMBER OF PAGES
25

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

A. Introduction

The long term objective of this project is to evaluate the capacity of a group of nonantimicrobial chemically modified tetracyclines to downregulate the angiogenic response which maintains breast tumor growth and proliferation. The elaboration of neovessels to supply nutrients to and remove metabolites from the growing tumor is generally ascribed to elaboration of soluble pro-angiogenic growth factors which trigger endothelial proliferation. Two angiogenic factors, Vascular Endothelial Growth Factor (VEGF) and Basic Fibroblast Growth Factor (b-FGF), have been implicated in breast tumor angiogenesis *in vivo* [1-5] and in studies of the pro-angiogenic activity of breast tumor cell lines as well as macrophages which often infiltrate tumors *in vivo* and are also believed to contribute to angiogenesis [6-9]. Recently, the National Cancer Institute has pursued clinical trials on one chemically modified tetracycline, 6-demethyl-6-deoxy-4-de(dimethylamino)tetracycline (CMT-3), a known inhibitor of the matrix metalloproteinase (MMP) class of enzymes implicated in cancer invasiveness [10], for use in therapy of a variety of tumors and the angioproliferative disease, Kaposi's sarcoma. The most promising results were observed with Kaposi's sarcoma patients, in several of whom complete regression of the microvascular lesions was observed [11]. It is this observation which prompted our proposal to examine the possibility that, in addition to their anti-MMP activity, the CMTs may also serve as antiangiogenic agents for treatment of breast cancer. Some studies indicating that CMT-3 may inhibit the angiogenic response of endothelial cells *in vitro* [12] and that other tetracyclines may be inhibitory in animal models of angiogenesis [13] have been published which support this hypothesis. The major adverse side effect of CMT-3 therapy (which is otherwise much less toxic than many traditional chemotherapeutic agents) observed during the course of the NCI trials has been phototoxicity, manifested as sunburn-like lesions [11]. In the course of evaluating new "second generation" CMTs synthesized for CollaGenex Pharmaceuticals, Inc., the manufacturer of CMT-3, we observed that the 9-amino derivative of CMT-3 (CMT-308) was equivalent to CMT-3 as an MMP inhibitor but did not trigger phototoxicity *in vitro* or in animal models [14]. This CMT was therefore included in our proposal as a possible additional candidate for antiangiogenic activity although it has not yet been evaluated in human subjects. Our approach in this project has been twofold: to evaluate the effects of the CMTs on the elaboration of pro-angiogenic growth factors by breast tumor cells and by infiltrating macrophages and to determine the effects on the response of endothelial cells to such angiogenic factors.

B. Body

This project has six Tasks which we have defined for study over the three-year period of support: to evaluate CMTs for effects on angiogenic factors released by breast tumor cell lines maintained under "basal" growth conditions (Task 1); to evaluate modulation of angiogenic factor release by the breast tumor cell lines by hormones, cytokines, and growth factors and to determine effects of the CMTs on these modulated levels of angiogenic factor release (Task 2); to evaluate effects of the CMTs on release of angiogenic factors by macrophages and a human macrophage line, Mono Mac 6 (Task 3); to evaluate capacity of the CMTs to inhibit tube formation and invasiveness of endothelial cells in response to pro-angiogenic factors (Task 4); to evaluate capacity of the CMTs to inhibit endothelial cell tube formation and invasiveness in response to breast tumor cell lines in a co-culture system (Task 5); and to evaluate capacity of the CMTs to inhibit endothelial cell tube formation and invasiveness in response to monocytoid cells in a co-culture system (Task 6). In this first year of the project, we have made progress on the first four of these Tasks.

1. *Task 1 - Effects of CMTs on VEGF Release by Breast Tumor Lines in the Absence of Growth Factors*

In this first year, we have focused on two breast tumor cell lines, MCF-7 (an estrogen-responsive line which is weakly invasive and is regarded as a model of early breast cancer) and MDA-MB-231 (an estrogen-insensitive line which is highly invasive and is regarded as a model of advanced breast cancer) [6]. In near confluent culture on plastic, both of these cell lines release VEGF, as detected by a sandwich ELISA, but no b-FGF. The levels of VEGF released by both cell lines were determined after 24 h of culture in complete medium followed by 24 hours of culture in serum-free medium in 48-well microplates. MCF-7 cells were plated at an initial density of 10^5

cells/well in MEM with supplements including 10% fetal bovine serum (FBS) while MDA-MB-231 cells were plated at the same density in DMEM with supplements including 10% FBS. At the end of 24 hours, when the medium was switched to serum-free medium, both cell lines were at 80-90% confluence. MCF-7 cells released approximately 40 pg/ml VEGF over 24 hours in the absence of any supplemental growth factors, whereas MDA-MB-231 cells released approximately 200 pg/ml VEGF over the same time (Figures 1-4). This result is consistent with observations that MDA-MB-231 cells metastasize much more effectively than MCF-7 cells in nude mice and may reflect the greater capacity of the MDA-MB-231 metastatic foci to establish a blood supply to support growth. Both CMT-3 and CMT-308 diminished the levels of VEGF released by these cell lines maintained under "basal" conditions in a dose-dependent manner. Neither CMT was especially cytotoxic to these breast tumor lines (Figures 5-7), a result which stands in marked contrast to the significant cytotoxicity of CMT-3 towards LNCaP and PC-3 prostate tumor lines [10]. The absence of cytotoxicity of CMT-308 on the two breast tumor lines is, however, consistent with its very low cytotoxicity in virtually every cell line tested so far in our laboratory. In the NCI-sponsored Phase I trials on CMT-3, circulating levels of the tetracycline on the order of 30 μM were routinely achieved [11], and while we tested levels of CMT-308 up to 30 μM in these *in vitro* assays of VEGF release, we generally employed lower concentrations of CMT-3, typically 5 μM, because of its cell-specific cytotoxic effects. However, in dose-dependence studies of both CMT-3 and CMT-308 on MCF-7 cells we have confirmed that at comparable doses up to 30 μM, CMT-308 is more effective at diminishing the levels of VEGF released than is CMT-3 (Figures 8 and 9). In the presence of 20 μM CMT-308, levels of VEGF released by MCF-7 cells are reduced by greater than 50%, whereas VEGF levels are reduced by only 17% in the presence of 20 μM CMT-3. The effects of the two CMTs on VEGF release by MDA-MB-231 cells in the absence of growth factors is qualitatively similar to the results outlined above on MCF-7 cells, and again, CMT-308 appears to be more potent than CMT-3.

2. Task 2 - Effects of Growth Factors on VEGF Release by Breast Tumor Lines in the Absence and Present of CMTs

In this first year, we have examined effects of two growth factors, TGF-β and IGF-1, on VEGF production by MCF-7 and MDA-MB-231 cells in the absence and presence of CMTs (neither of these growth factors appeared to induce b-FGF release by the breast tumor cell lines). These data are also included in Figures 1-4. In preliminary studies, CMT-308 was found to diminish levels of MMP-9 released by PC-3 prostate tumor cells, but in the presence of IGF-1, the MMP-9 levels were augmented. The increase in MMP-9 from IGF-1-stimulated prostate tumor cells treated with CMT-308 may have possibly arisen through reduction in the levels of the IGF-1-neutralizing binding protein IGFBP-3 which is released by MMPs in other prostate tumor cell lines [15,16]. In the presence of TGF-β, however, levels of MMP-9 released by PC-3 cells were found to be augmented and upon addition of CMT-308, the MMP-9 levels were lowered towards those released in the absence of growth factors. VEGF levels released by MCF-7 cells and MDA-MB-231 cells were unaffected by doses of IGF-1 up to 10 ng/ml, and the capacity of 20 μM CMT-308 to diminish VEGF release by both cell lines in the presence of different concentrations of IGF-1 was the same as that observed in the absence of growth factors within the limits of experimental error (Figures 2 and 4). We conclude that IGF-1 does not modulate VEGF release by these breast tumor cell lines. In contrast, TGF-β was a significant stimulus to VEGF release by both cell lines. VEGF levels from MDA-MB-231 cells were tripled in the presence of 10 ng/ml TGF-β; the levels from MCF-7 cells were increased somewhat less, by a factor of about 2.5. CMT-308 at a concentration of 20 μM appeared to be about as effective at inhibiting this TGF-β-induced augmented VEGF release as was seen when VEGF release by the breast tumor cells was measured in the absence of growth factors while concentrations of CMT-3 as low as 5 μM, which had minimal effect on VEGF release by the cells in the absence of growth factors, produced a larger and experimentally significant diminution in the augmented levels of VEGF produced in the presence of TGF-β (Figures 1 and 3). We conclude that TGF-β stimulates VEGF production by these two cell lines, and that the two CMTs can inhibit this augmented release at least as effectively as they inhibit "baseline" levels of VEGF released by the lines in the absence of growth factors.

3. Task 3 - *Effects of CMTs on VEGF Release by Mono Mac 6 Cells*

The Mono Mac 6 cell line was developed by Ziegler-Heitbrock's laboratory from a patient monocytic leukemia, and, while it is immortal, it possesses many characteristics of unactivated circulating monocytes [17]. We have previously shown that Mono Mac 6 cells release very low levels of MMP-9 unless they are stimulated with an activating agent such as phorbol myristate acetate (PMA), but in the presence of CMTs, the augmented MMP-9 levels are diminished back towards the "baseline" levels. Our standard conditions for maintaining Mono Mac 6 which we employed for the studies in this project employ a culture medium consisting of RPMI supplemented with oxaloacetic acid, pyruvate, insulin, and 10% FBS. As in the case of the breast tumor lines, we maintained the Mono Mac 6 cells in 48 well plates at a density of 10^5 per well for 24 hours in complete medium which was then replaced by RPMI alone at the time of addition of CMTs. Culture was continued for 24 or 48 hours prior to collection of medium for measurement of angiogenic factors. In this first year of this project, we observed that addition of either TGF-β or IGF-1 at concentrations as high as 10 ng/ml to Mono Mac 6 cells had no effect on VEGF levels released by the cells, while no b-FGF release could be detected under any conditions (Figures 10 and 11). The levels of VEGF released by 10^5 unstimulated Mono Mac 6 cells/well were about comparable to those released by MCF-7 cells - approximately 40 pg/ml in 24 hours; in 48 hours, the levels of VEGF had increased to 120 pg/ml. As had been observed with the breast tumor cell lines, addition of CMT-3 resulted in only modest diminution of VEGF levels released by the Mono Mac 6 cells; in the presence of 20 μM CMT-3 the levels were diminished by somewhat less than 50% (Figure 12). Some of this reduction at higher CMT-3 levels may simply be due to cytotoxicity. CMT-308, in contrast, had extremely potent effects on VEGF release by Mono Mac 6 cells. Diminution of greater than 50% was seen in the presence of 5 μM CMT-308, while in the presence of higher concentrations of this CMT, VEGF levels were virtually undetectable (Figure 12). As observed with the other cell lines, concentrations of CMT-308 as great as 20 μM had no cytotoxic effect on the Mono Mac 6 cells. We conclude that CMT-308, but not CMT-3, is an extremely potent inhibitor of VEGF release by Mono Mac 6 cells, reducing VEGF levels to below the limit of detection at concentrations of 10 and 20 μM, but neither TGF-β nor IGF-1 affects VEGF levels in the absence or presence of the CMTs. If the hypothesis is valid that infiltrating "tumor-associated macrophages" may contribute to the pro-angiogenic signals released by tumors [18], CMT-308 may prove to be unusually effective as an anti-angiogenic agent.

4. *Task 4 - Effects of CMTs on Human Endothelial Cells*

In this first year of the project, we have made only preliminary progress on the effects of the CMTs on endothelial cells. Human umbilical vein endothelial cells were obtained from Clonetics Corp. and grown to confluence in 48 well microplates using the company's proprietary growth medium. In the presence of CMT-3 or CMT-308 at concentrations up to 20 μM, we observed no loss of viability of these confluent cells as judged by reduction of the tetrazolium salt MTS to its formazan (Figure 13). We have not yet evaluated whether the CMTs have any cytotoxic effects on endothelial cells which are undergoing proliferation, migration, invasion, or tube formation. Our preliminary studies on endothelial cell invasion through Matrigel in response to the pro-angiogenic agent VEGF were carried out with human umbilical vein cells as well, but these cells have been reported to show much less invasiveness than microvascular endothelial cells. The experimental system we intend to employ for these assays is based on the use of Matrigel-coated membrane-bottomed inserts for multiwell microplates with a new modification, developed by Becton Dickinson Corp. The membrane bottoms of the inserts are impregnated with a purple dye which absorbs light in the wavelength range where fluorescein and its analogs emit. The endothelial cells are allowed to take up a fluorescein-like vital stain after migration through the membranes in response to a VEGF signal and the number of cells which have reached the undersurface of the membrane can then be quantitated by measuring fluorescence in the Cytofluor 2300 microplate fluorimeter, which reads plates from the undersurface. We have confirmed the feasibility of measurements with this technique using the dye calcein-AM which is trapped within viable cells by cytosolic nonspecific esterases. This technology will be employed for evaluating migrating, invading, and tube forming microvascular endothelial cell responses to the CMTs as well as effects of pre-incubation with CMTs on the subsequent capacity of the endothelial cells to migrate, invade, and form tubes in thick matrices.

C. Key Research Accomplishments - 5/15/01-5/14/02

- Determined levels of VEGF released by MCF-7 (a model of early breast cancer) and MDA-MB-231 (a model of advanced breast cancer) cell lines. The MDA-MB-231 cells release higher levels of VEGF than the MCF-7 cells on a per cell basis in the absence of supplemental growth factors. Neither cell line releases detectable levels of b-FGF.
- Demonstrated inhibition of VEGF release from MCF-7 cells and MDA-MB-231 cells cultured in the presence of CMT-3 or CMT-308. At equivalent doses, CMT-308 inhibits VEGF release from both cell lines significantly more than CMT-3. Inhibition of VEGF release is detected at doses which are not cytotoxic to either cell line.
- Demonstrated augmented release of VEGF from MCF-7 cells and MDA-MB-231 cells cultured in the presence of added TGF-β; this increase in levels of VEGF is dependent upon the levels of added TGF-β.
- Demonstrated that the augmented levels of VEGF released by MCF-7 and MDA-MB-231 cell lines in the presence of TGF-β are diminished somewhat upon addition of CMT-3 and more significantly upon addition of CMT-308.
- Demonstrated that addition of IGF-1 to MCF-7 and MDA-MB-231 cell lines has no effect on VEGF release.
- Demonstrated that the human monocytic cell line Mono Mac 6 (a model of tumor infiltrating macrophages) releases VEGF in the absence of added stimuli; VEGF levels from this cell line are unaltered in the presence of TGF-β or IGF-1.
- Demonstrated that CMT-3 has minimal effect on VEGF release from Mono Mac 6 cells but CMT-308 is a very potent inhibitor of VEGF release, diminishing VEGF to virtually undetectable levels in the absence of cytotoxicity.
- Demonstrated that CMT-3 and CMT-308 are not cytotoxic to confluent human umbilical vein endothelial cells at doses which inhibit VEGF release from the two tested breast tumor cell lines.

D. Reportable Outcome

In this first year, we have only one reportable outcome, the abstract of the work to be presented in this September's USAMRMC Era of Hope 2002 Breast Cancer Research Program. The abstract, entitled "Antiangiogenic Action of Chemically Modified Tetracyclines in Breast Cancer," by Sanford R. Simon and Mansi Kothari, is included as an appendix to this report.

E. Conclusions

In the first year of research on this project we do not wish to overstate the implications of the results obtained so far, but we are especially encouraged by the marked reduction in VEGF release from Mono Mac 6 cells in the presence of doses of CMT-308 as low as 5 μM, as well as the less marked but still significant diminutions in release of VEGF from the two breast tumor cell lines we have evaluated in the presence of higher, but still nontoxic, doses of CMT-308. If infiltration of tumors by macrophages proves to be an important feature of the mechanism underlying the generation of signals favoring tumor angiogenesis, the possibility that this CMT can be effective at reducing levels of a recognized angiogenic factor from both tumor cells and infiltrating macrophages may favor its use an antiangiogenic agent in management of breast cancer.

F. References

1. Relf, M., LeJeune, S., Scott, P.A., Fox, S., Smith, K., Leek, R., Moghaddam, A., Whitehouse, R., Bicknell, R., and Harris, A.L. (1997) Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet derived entohelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res. 57:963-969.

2. Eppenbeger, U., Kueng, W., Schlaeppi, J.M., Roesel, J.L., Benz, C., Mueller, H., Matter, A., Zuber, M., Luescher, K., Litschi, M., Schmitt, M., Foekens, J.A., and Eppenberger-Castori, S. (1998) Markers of tumor angiogenesis and proteolysis independently define high- and low-risk subsets of node-negative breast cancer patients. J. Clin. Oncol. 16:3129-3136.

3. Lichtenbeld, H.C., Barendsz-Janson, A.F., van Essen, H., Struiker Boudier, H., Griffioen, A.W., and Hillen, H.F. (1998) Angiogenic potential of malignant and non-malignant human breast tissues in an in vivo angiogenesis model. Intern. J. Cancer 77:455-459.

4. de Jong, J.S., van Diest, P.J., van der Valk, P., and Baak, J.P. (1998) Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlation with proliferation and angiogenesis. J. Pathol. 184:53-57.

5. Speirs, V., and Atkin, S.L. (1999) Production of VEGF and expression of the VEGF receptors Flt-1 and KDR in primary cultures of eipthelial and stromal cells derived from breast tumors. Brit. J. Cancer 80:898-903.

6. Scott, P.A.E., Gleadle, J.M., Bicknell, R., and Harris, A.L. (1998) Role of the hypoxia sensing system, acidity and reproductive hormones in the variability of vascular endothelial growth factor induction in human breast carcinoma cell lines. Intern. J. Cancer. 75:706-712.

7. Goede, V. Brogelli, L., Ziche, M., and Augustin, H.G. (1999) Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. Intern. J. Cancer 82:765-770.

8. Donovan, D., Harmey, J.H., Toomey, D., Osborne, D.H., Redmond, H.P., and Bouchier-Hayes, D.J. (1997) TGF beta-1 regulation of VEGF production by breast cancer cells. Ann. Surg. Oncol. 4:621-627.

9. Ruohola, J.K., Valve, E.M., Karkkainen, M.J., Joukov, V., Alitalo, K., and Harkonen, P.L. (1999) Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Molec. Cell. Endocrin. 149:29-40.

10. Gu, Y., Lee, H.M., Roemer, E.J., Musacchia, L., Golub, L.M., and Simon, S.R. (2001) Inhibition of tumor cell invasiveness by chemically modified tetracyclines. *Curr. Med. Chem.* 8:261-270.

11. Cianfrocca, M., Cooley, T.P., Lee, J.Y., Rudek, M.A., Scadden, D.T., Ratner, L., Pluda, J.M., Figg, W.D., Krown, S.E., and Dezube, B.J. (2002) Matrix metalloproteinase inhibitor COL-3 in the treatment of AIDS-related Kaposi's sarcoma: a phase I AIDS malignancy consortium study. J. Clin. Oncol. 20:153-159.

12. Fife, R.S., Sledge, G.W., Sissons, S., and Zerler, B. (2000) Effects of tetracyclines on angiogenesis in vitro. Cancer Lett. 153:75-78.

13. Tamargo, R.J., Bok, R.A., and Brem, H. (1991) Angiogenesis inhibition by minocycline. Cancer Res. 51:672-675.

14. Zerler, B., Roemer, E., Raabe, H., Sizemore, A., Reeves, A., and Harbell, J. (2000) Evaluation of the phototoxic potential of chemically modified tetracyclines with the 3T3 neutral red uptake phototoxicity test. Progr. Reduct. Refine. Replace. Animal Exper., Balls, M, van Zeller, A., and Halder, M., eds. pp. 545-554.

15. Simon, S.R. (2001) Inhibition of prostate tumor cell invasiveness by chemically modified tetracyclines with broad spectrum antiproteinase activity. USAMRMC CDMRP PCRP DAMD17-98-1-8560 Final Report.

16. Manes, S., Llorente, M., Lacalle, R.A., Gomez-Mouton, C., Kremer, L., Mia, E., and Martinez, C. (1999) The matrix metalloproteinase-9 regulates the insulin-like growth factor-triggered autocrine response in DU-145 carcinoma cells. J. Biol. Chem. 274:6935-6945.

17. Ziegler-Heitbrock, H.W., Schraut, W., Wendelgass, P., Strobel, M., Sternsdorf, T., Weber, C., Aepfelbacher, M., Ehlers, M., Schutt, C., and Haas, J.G. (1994) Distinct patterns of differentiation induced in the monocytic cell line, Mono Mac 6. J. Leukocyte Biol. 55:73-80.

18. Lewis, C.E., Leek, R., Harris, A., and McGee, J.O. (1995) Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. J. Leukocyte Biol. 57:747-751.

Figures

1. Effects of CMTs on MCF-7 cells stimulated with TGF-β. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 0.1, 1.0, or 10.0 ng/ml TGF-β, and no CMTs or 5 μM CMT-300 or 20 μM CMT-308. After the 24 hour incubation in serum-free medium, the supernatants were collected for assay of VEGF by ELISA and the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

2. Effects of CMTs on MCF-7 cells stimulated with IGF-1. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 0.1, 1.0, or 10.0 ng/ml IGF-1, and no CMTs or 5 μM CMT-300 or 20 μM CMT-308. After the 24 hour incubation in serum-free medium, the supernatants were collected for assay of VEGF by ELISA and the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

3. Effects of CMTs on MDA-MB-231 cells stimulated with TGF-β. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 0.1, 1.0, or 10.0 ng/ml TGF-β, and no CMTs or 5 μM CMT-300 or 20 μM CMT-308. After the 24 hour incubation in serum-free medium, the supernatants were collected for assay of VEGF by ELISA and the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

4. Effects of CMTs on MDA-MB-231 cells stimulated with IGF-1. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 0.1, 1.0, or 10.0 ng/ml IGF-1, and no CMTs or 5 μM CMT-300 or 20 μM CMT-308. After the 24 hour incubation in serum-free medium, the supernatants were collected for assay of VEGF by ELISA and the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

5. Cytotoxicity of CMT-300 and CMT-308 to MCF-7 cells. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 5, 10, 20, or 30 μM CMT-3 or CMT-308 in the absence of added growth factors. After the 24 hour incubation in serum-free medium, the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

6. Cytotoxicity of CMT-300 to MDA-MB-231 cells. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 1, 3, 5, or 10 μM CMT-3 in the absence of added growth factors. After the 24 hour incubation in serum-free medium, the cells were incubated with the tetrazolium salt MTS for a period of 4 hours for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer every hour over the 4 hour incubation.

7. Cytotoxicity of CMT-308 to MDA-MB-231 cells. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 1, 10, 20, or 30 μM CMT-308 in the absence of added growth factors. After the 24 hour incubation in serum-free medium, the cells were incubated with the tetrazolium salt MTS for a period of 4 hours for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer every hour over the 4 hour incubation.

8. Dose-dependent inhibition of VEGF release from MCF-7 cells by CMT-300. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 5, 10, 20, or 30 μM CMT-3 in the presence of 0, 0.1, 1.0, or 10.0 ng/ml TGF-β. After the 24 hour incubation in serum-free medium, the supernatants were collected for assay of VEGF by ELISA.

9. Dose-dependent inhibition of VEGF release from MCF-7 cells by CMT-308. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh serum-free medium containing 0, 5, 10, 20, or 30 μM CMT-308 in the presence of 0, 0.1, 1.0, or 10.0 ng/ml TGF-β. After the 24 hour incubation in serum-free medium, the supernatants were collected for assay of VEGF by ELISA.

10. Effects of CMTs on VEGF release from Mono Mac 6 cells in the absence and presence of TGF-β. After 24 hours of culture in complete medium, the cells were incubated for an additional 48 hours in fresh medium containing 0, 0.1, 1.0, or 10.0 ng/ml TGF-β, and no CMTs or 5 μM CMT-300 or 20 μM CMT-308. After the 48 hour incubation in medium containing inhibitors and/or TGF-β, the supernatants were collected for assay of VEGF by ELISA and the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

11. Effects of CMTs on VEGF release from Mono Mac 6 cells in the absence and presence of IGF-1. After 24 hours of culture in complete medium, the cells were incubated for an additional 48 hours in fresh medium containing 0, 0.1, 1.0, or 10.0 ng/ml IGF-1, and no CMTs or 5 μM CMT-300 or 20 μM CMT-308. After the 48 hour incubation in medium containing inhibitors and/or IGF-1, the supernatants were collected for assay of VEGF by ELISA and the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

12. Dose-dependent inhibition of VEGF release from Mono Mac 6 cells by CMT-308. After 24 hours of culture in complete medium, the cells were incubated for an additional 24 hours in fresh medium containing 0, 5, 10, or 20 μM CMT-3 or CMT-308 in the absence of added growth factors. After the 24 hour incubation in medium containing inhibitors, the supernatants were collected for assay of VEGF by ELISA and the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

13. Cytotoxicity of CMTs towards human umbilical vein endothelial cells (HUVECs). HUVECs were supplied by Clonetics Corp. and maintained in Clonetics Endothelial Growth Medium until confluent. The medium was then replaced with fresh medium containing 0, 1, 10, 50, or 100 μM CMT-3 or CMT-308 and incubation was continued for an additional 24 hours. After the 24 hour incubation in medium containing inhibitors, the cells were incubated with the tetrazolium salt MTS for determination of viability. Conversion of MTS to formazan was quantitated by measuring absorbance at 490 nm in a multiwell microplate spectrometer.

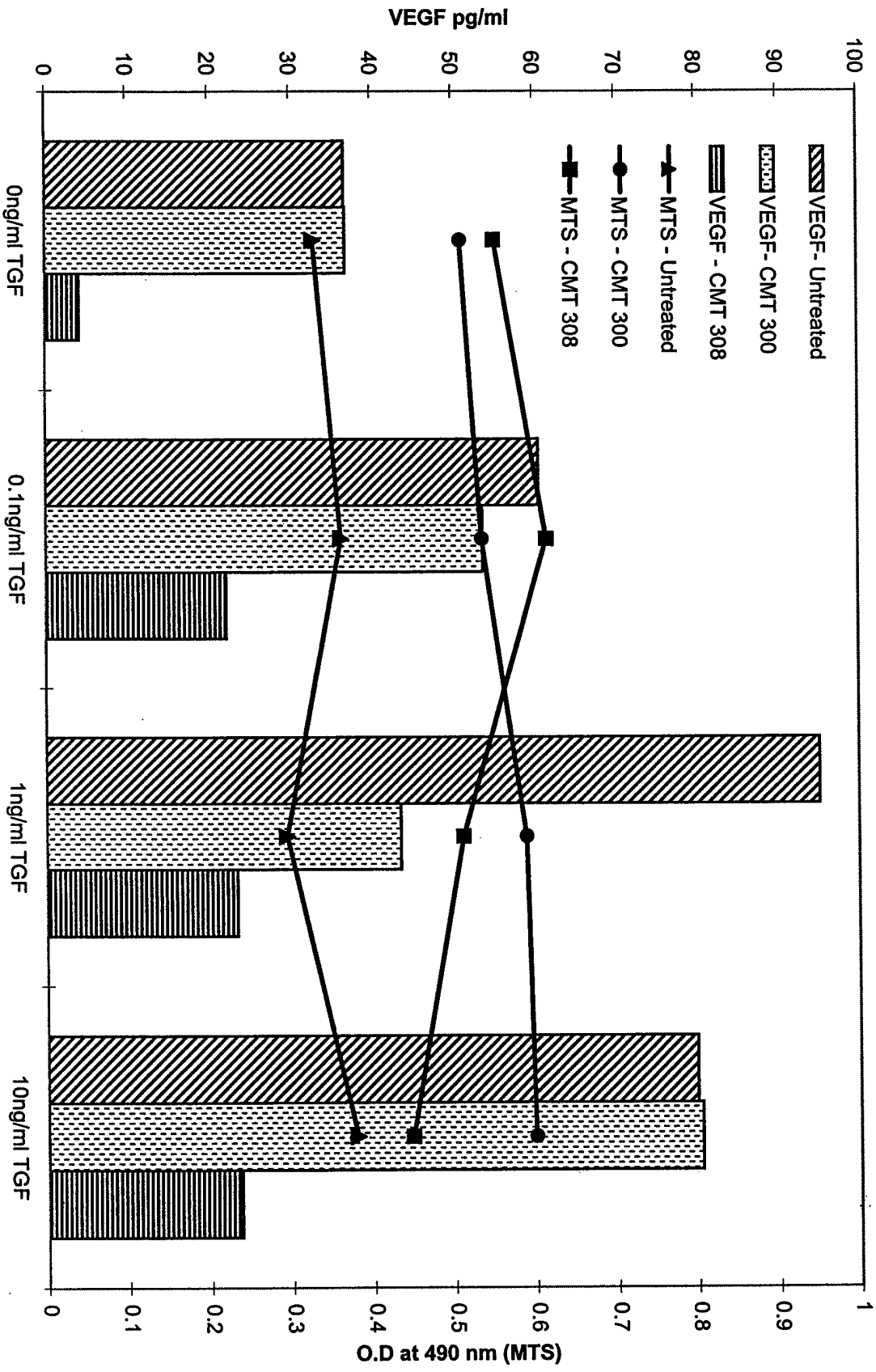
Effect of CMTs on MCF-7 cells stimulated with TGF Beta
VEGF pg/ml
O.D at 490 nm (MTS)
VEGF- Untreated
VEGF- CMT 300
VEGF - CMT 308
MTS - Untreated
MTS - CMT 300
MTS - CMT 308
0ng/ml TGF
0.1ng/ml TGF
1ng/ml TGF
10ng/ml TGF
0
10
20
30
40
50
60
70
80
90
100
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1

Figure 1

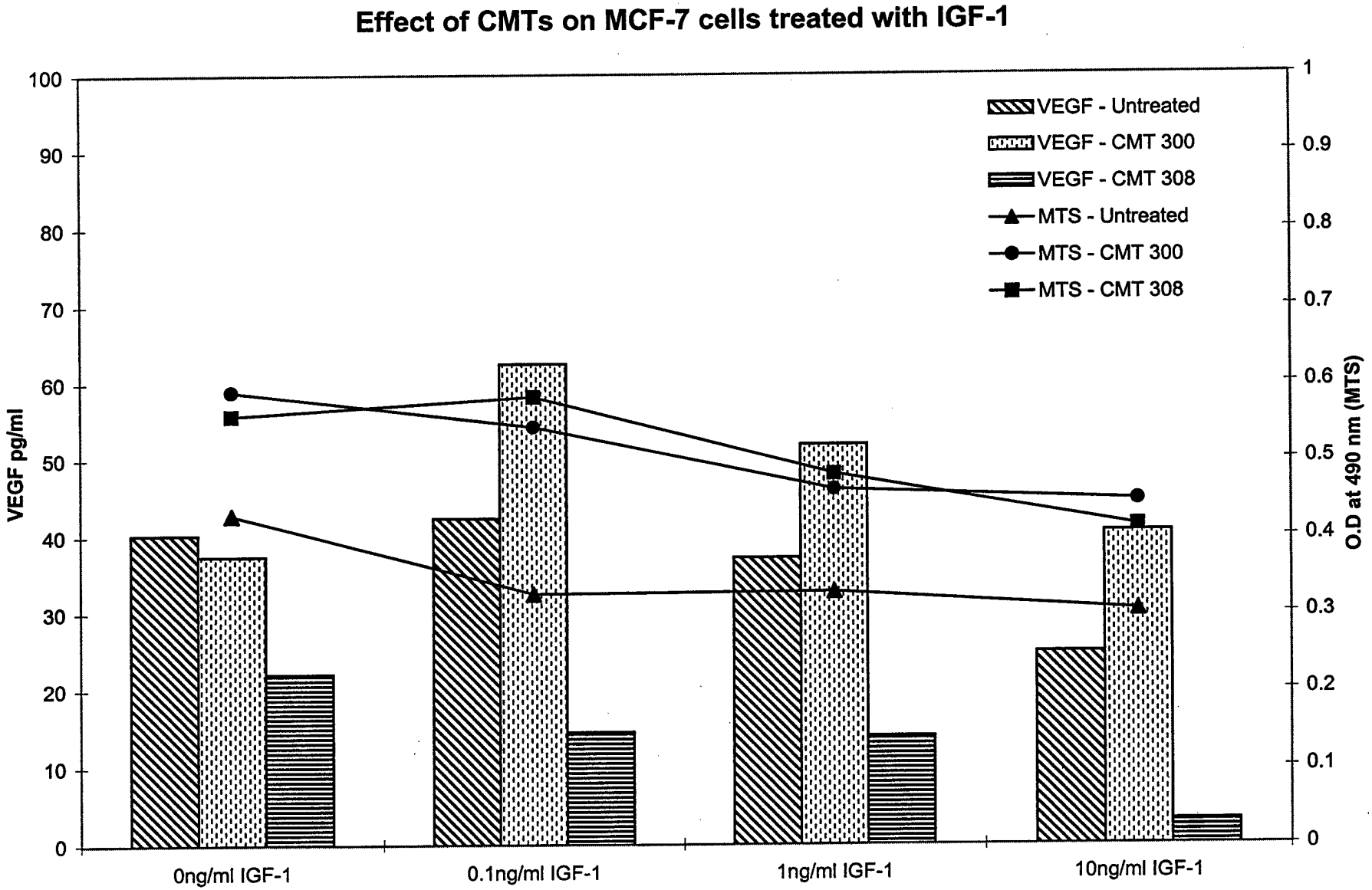

Figure 2
Page 1

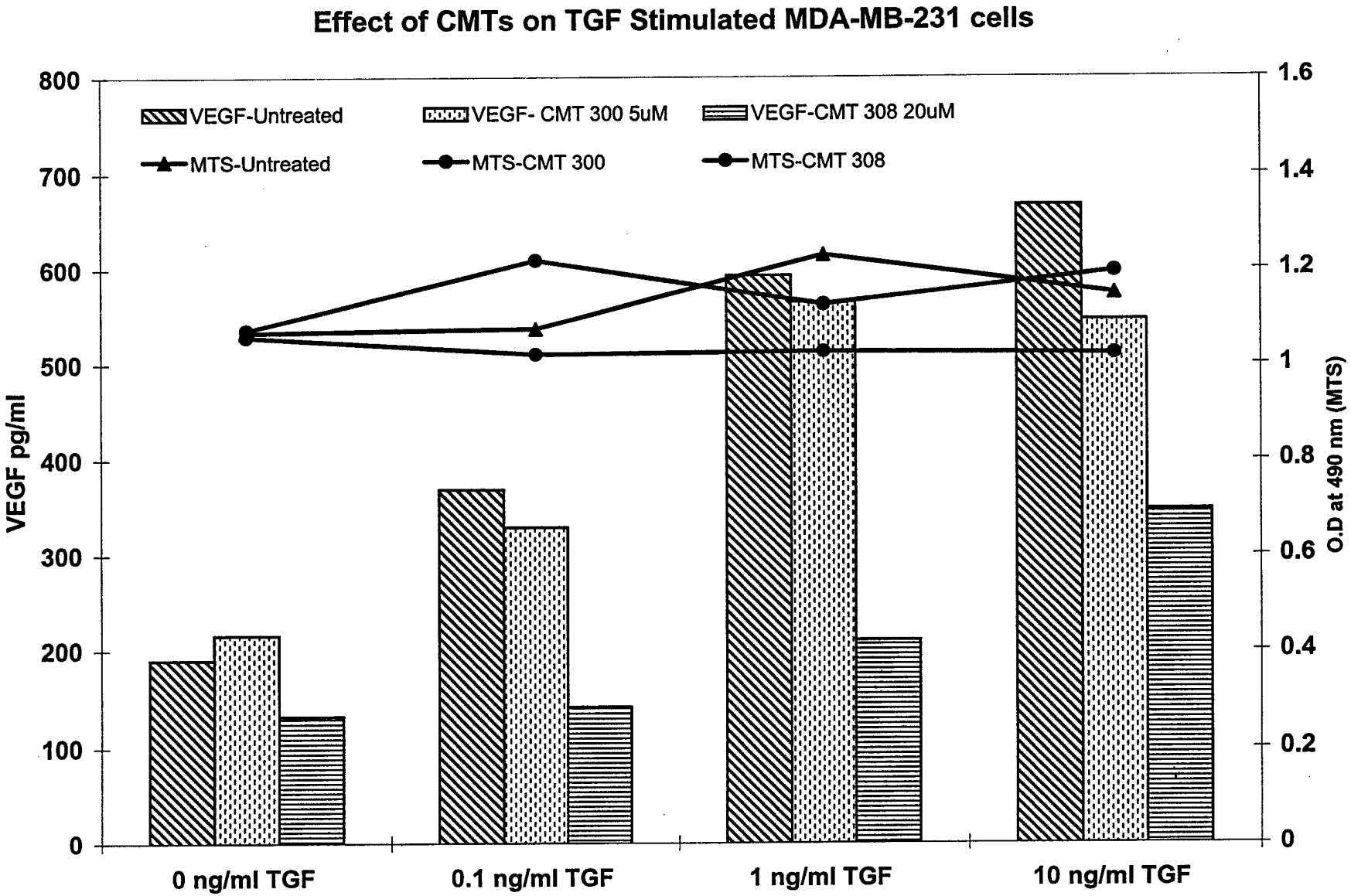
Effect of CMTs on TGF Stimulated MDA-MB-231 cells
VEGF-Untreated
VEGF- CMT 300 5uM
VEGF-CMT 308 20uM
MTS-Untreated
MTS-CMT 300
MTS-CMT 308
VEGF pg/ml
O.D at 490 nm (MTS)
800
700
600
500
400
300
200
100
0
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 ng/ml TGF
0.1 ng/ml TGF
1 ng/ml TGF
10 ng/ml TGF

Figure 3

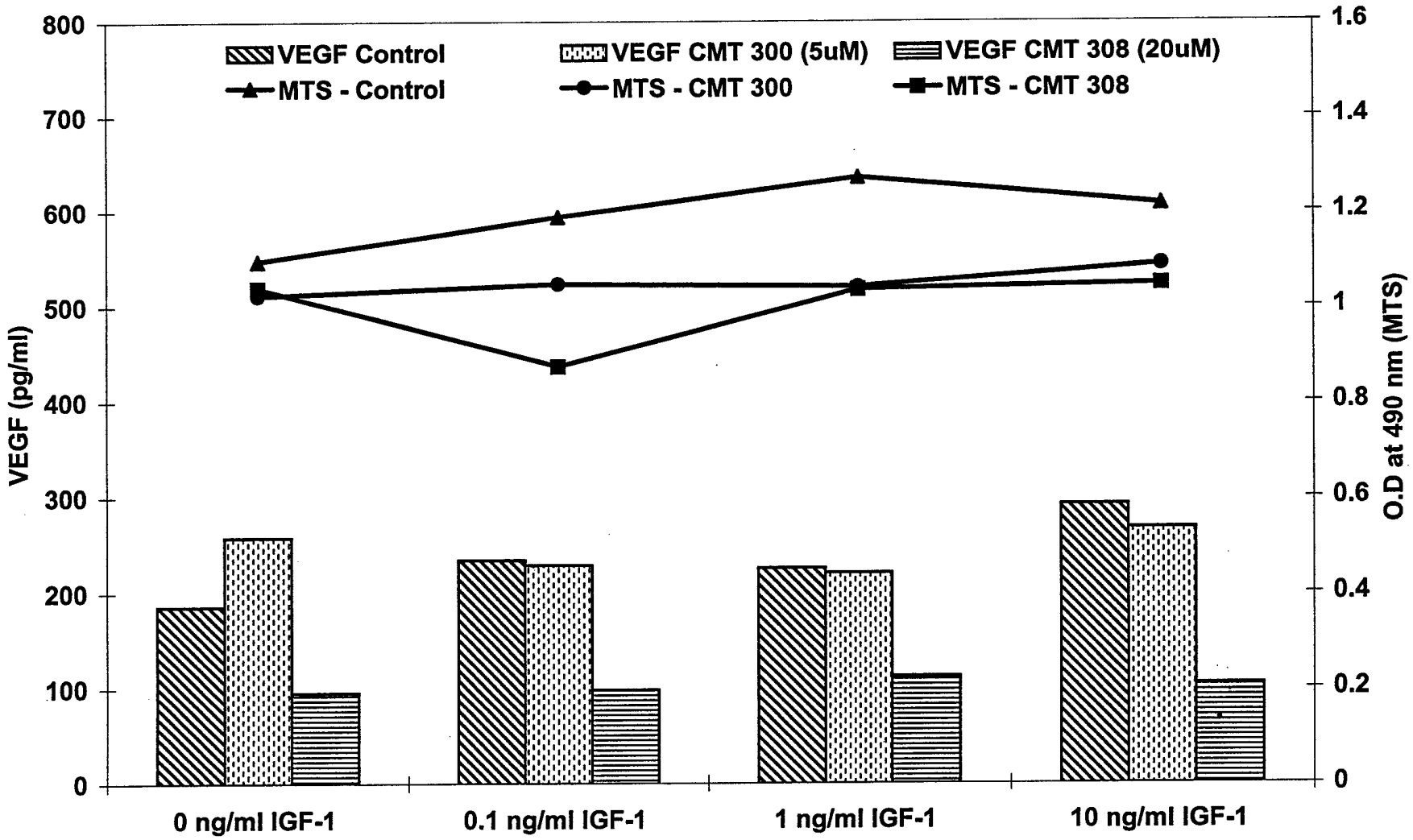
Effect of CMTs on IGF-I treated MDA-MB-231 cells
VEGF Control
VEGF CMT 300 (5uM)
VEGF CMT 308 (20uM)
MTS - Control
MTS - CMT 300
MTS - CMT 308
VEGF (pg/ml)
O.D at 490 nm (MTS)
800
700
600
500
400
300
200
100
0
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 ng/ml IGF-1
0.1 ng/ml IGF-1
1 ng/ml IGF-1
10 ng/ml IGF-1

Figure 4

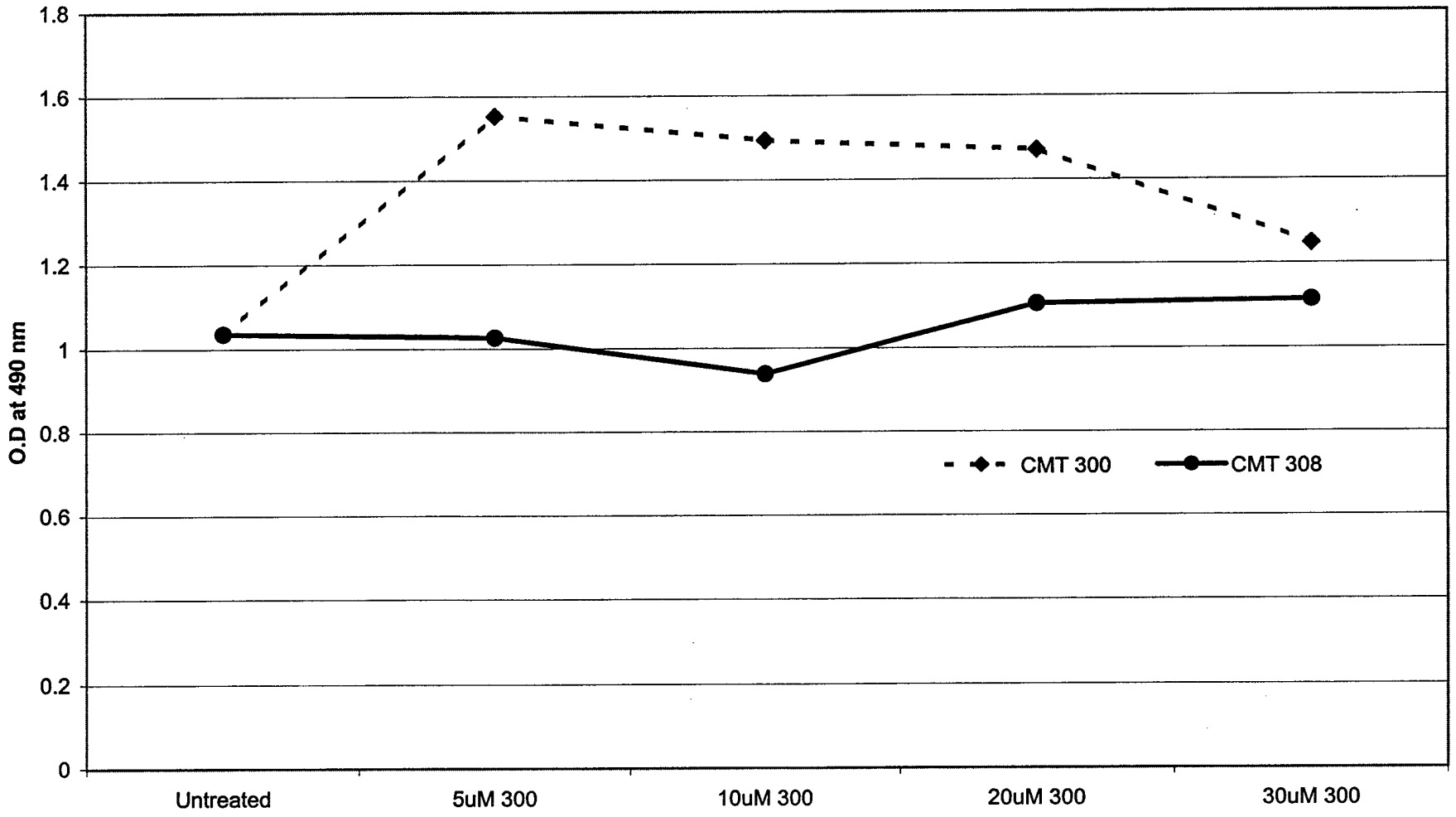
Cytotoxicity of CMTs on MCF-7 cells
O.D at 490 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT 300
CMT 308
Untreated
5uM 300
10uM 300
20uM 300
30uM 300

Figure 5

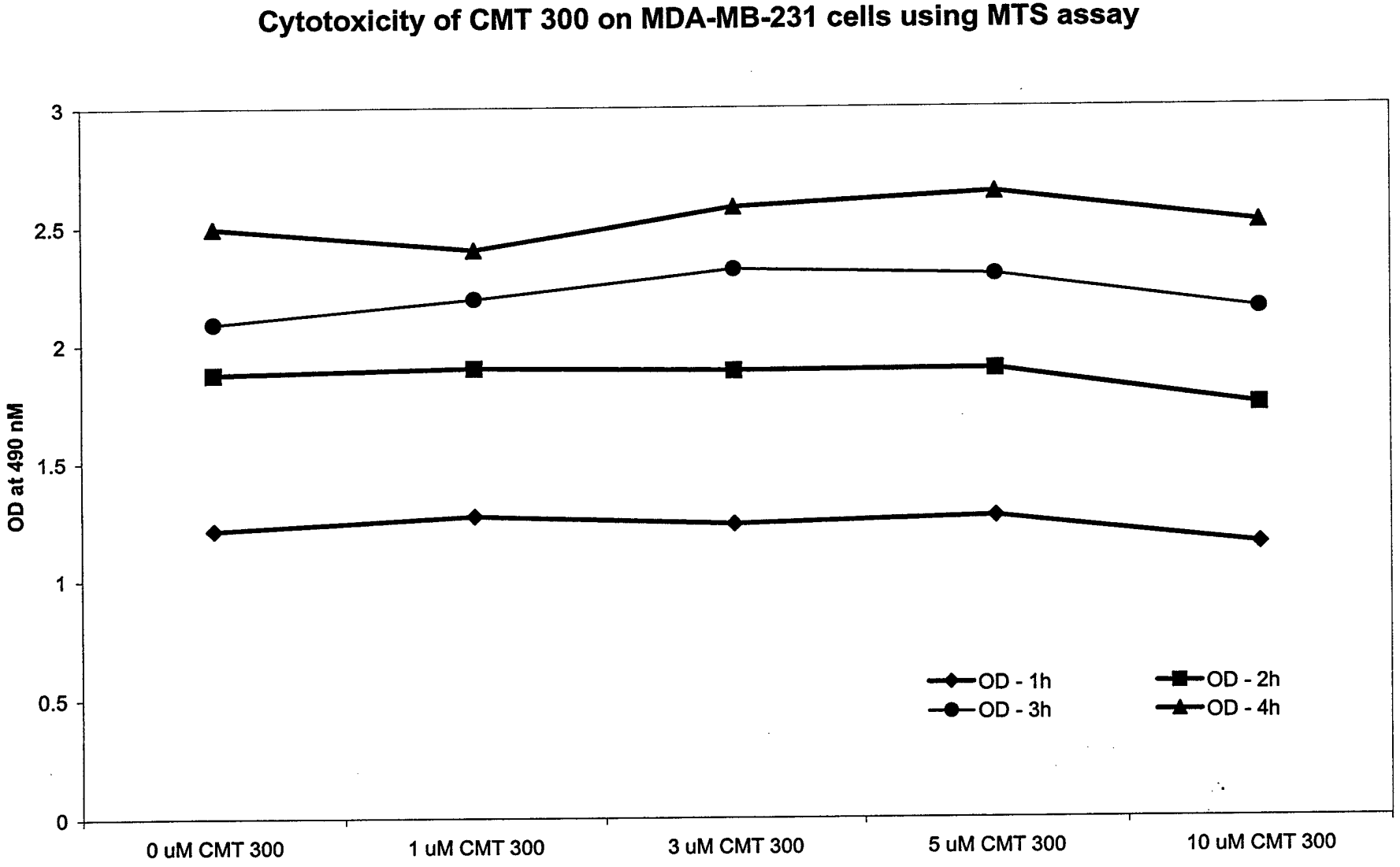
Cytotoxicity of CMT 300 on MDA-MB-231 cells using MTS assay
OD at 490 nM
3
2.5
2
1.5
1
0.5
0
0 uM CMT 300
1 uM CMT 300
3 uM CMT 300
5 uM CMT 300
10 uM CMT 300
OD - 1h
OD - 2h
OD - 3h
OD - 4h

Figure 6

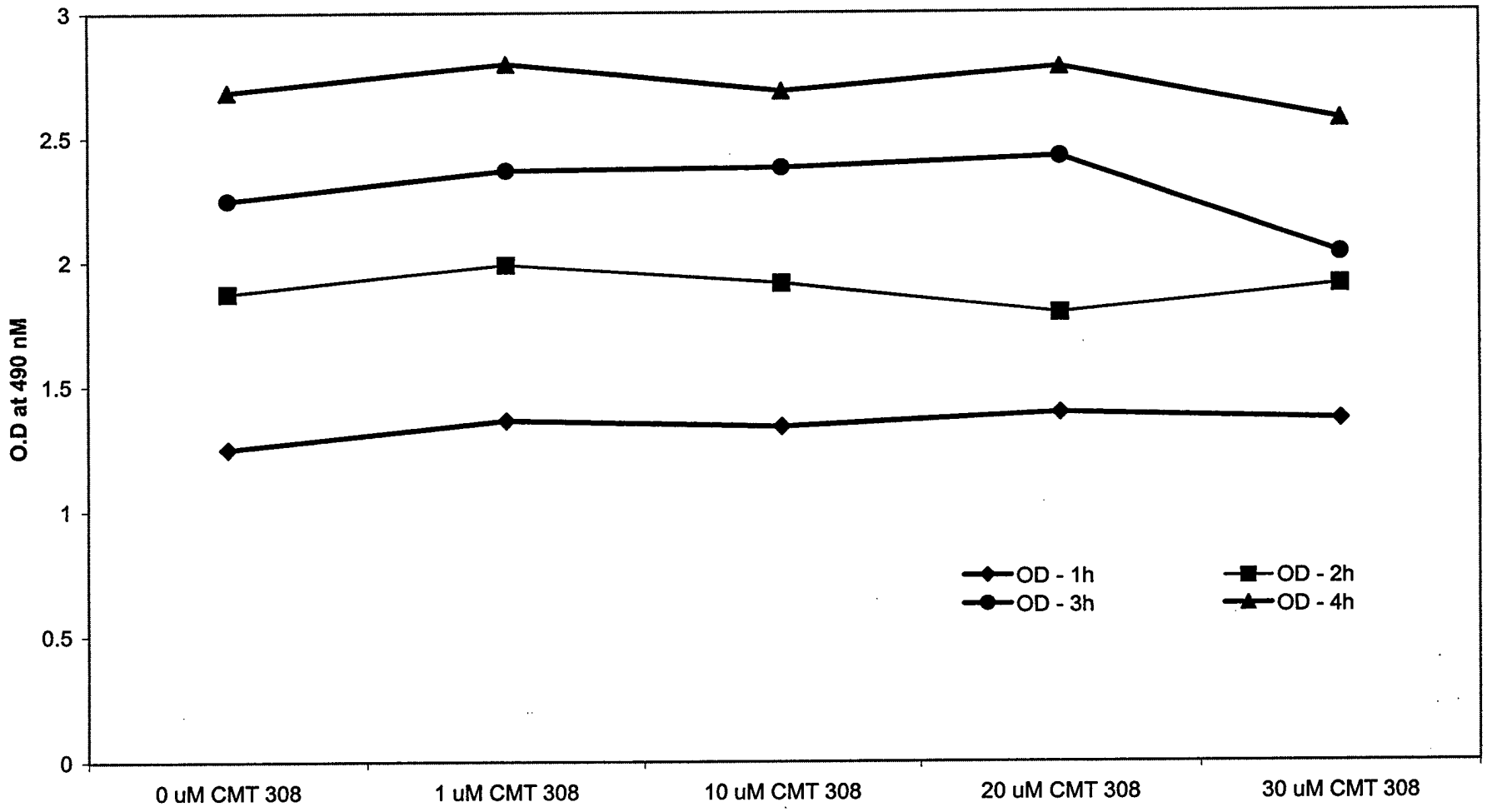
Cytotoxic Effect of CMT 308 on MDA-MB-231 cells using MTS Assay
O.D at 490 nM
3
2.5
2
1.5
1
0.5
0
0 uM CMT 308
1 uM CMT 308
10 uM CMT 308
20 uM CMT 308
30 uM CMT 308
OD - 1h
OD - 2h
OD - 3h
OD - 4h

Figure 7

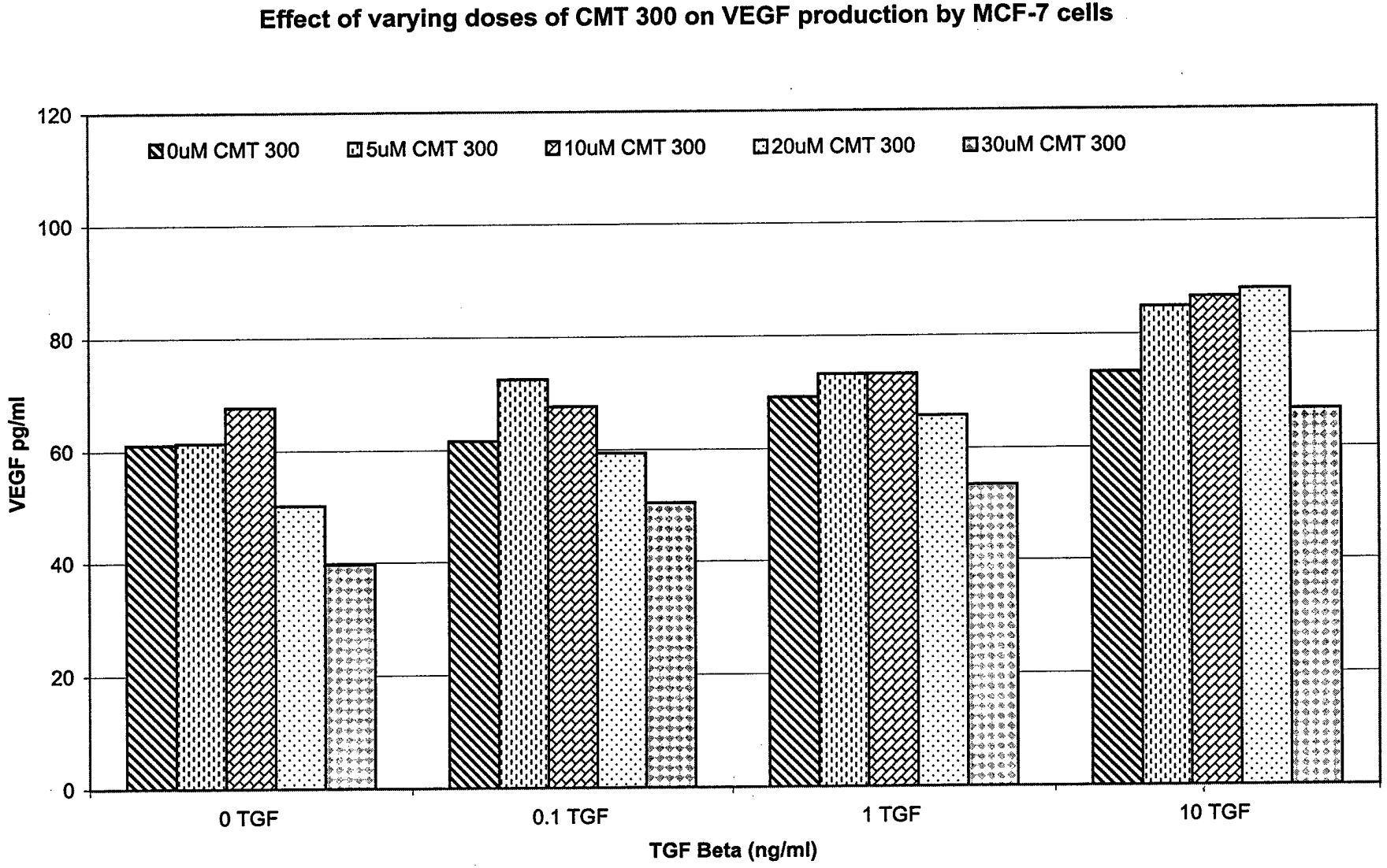
Effect of varying doses of CMT 300 on VEGF production by MCF-7 cells
0uM CMT 300
5uM CMT 300
10uM CMT 300
20uM CMT 300
30uM CMT 300
VEGF pg/ml
120
100
80
60
40
20
0
0 TGF
0.1 TGF
1 TGF
10 TGF
TGF Beta (ng/ml)

Figure 8

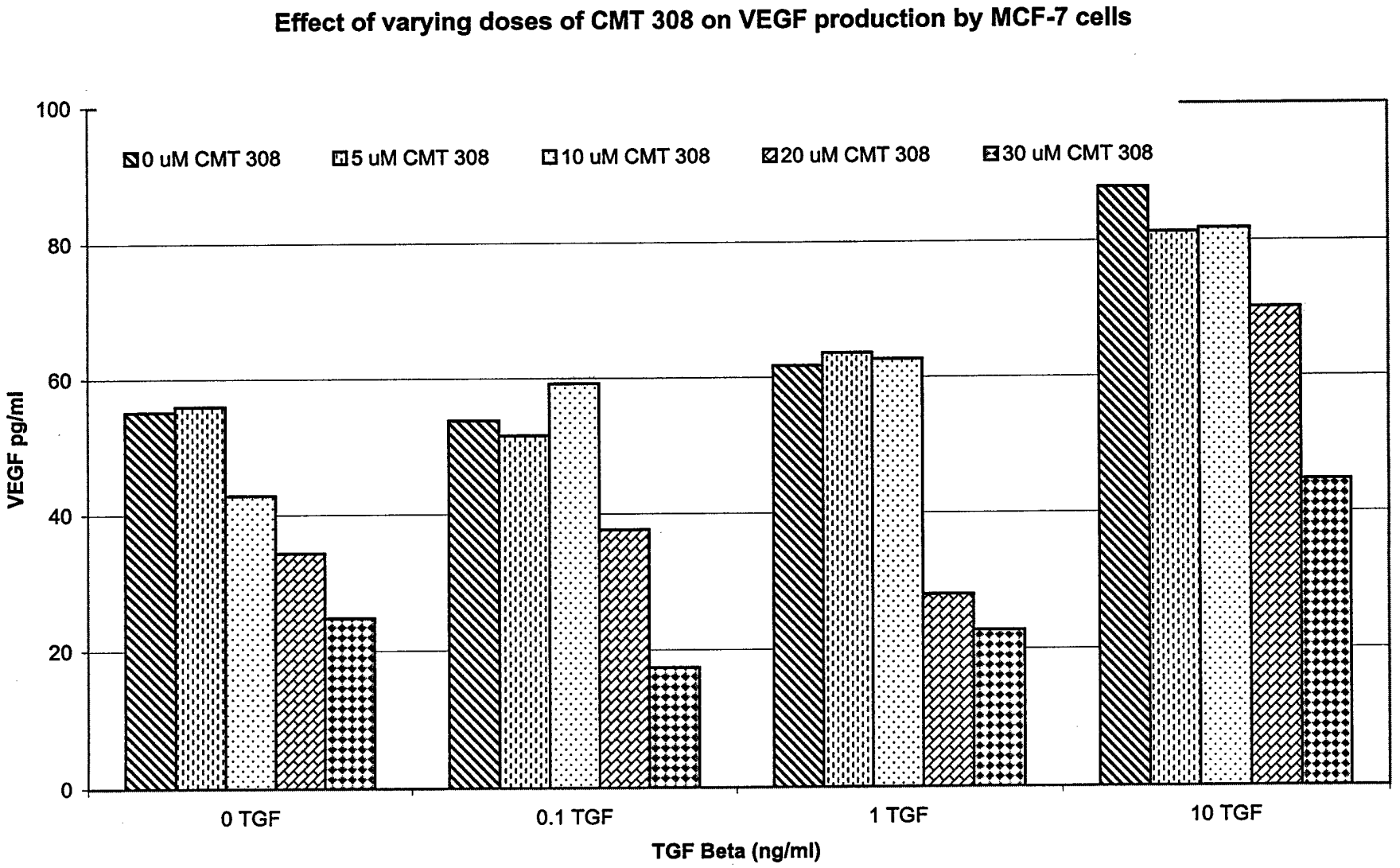
Effect of varying doses of CMT 308 on VEGF production by MCF-7 cells
100
80
60
40
20
0
VEGF pg/ml
0 uM CMT 308
5 uM CMT 308
10 uM CMT 308
20 uM CMT 308
30 uM CMT 308
0 TGF
0.1 TGF
1 TGF
10 TGF
TGF Beta (ng/ml)

Figure 9

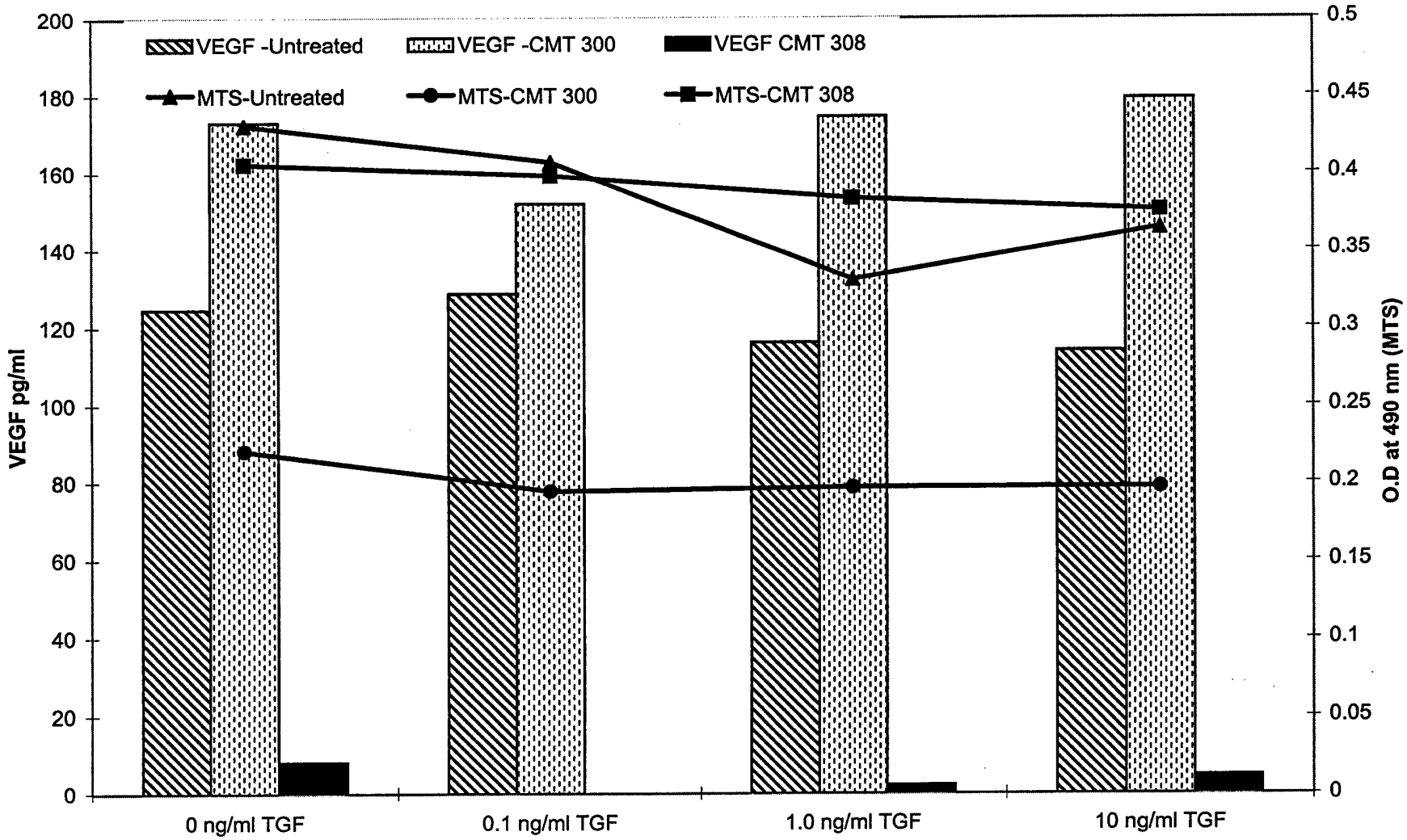

Figure 10
Page 1

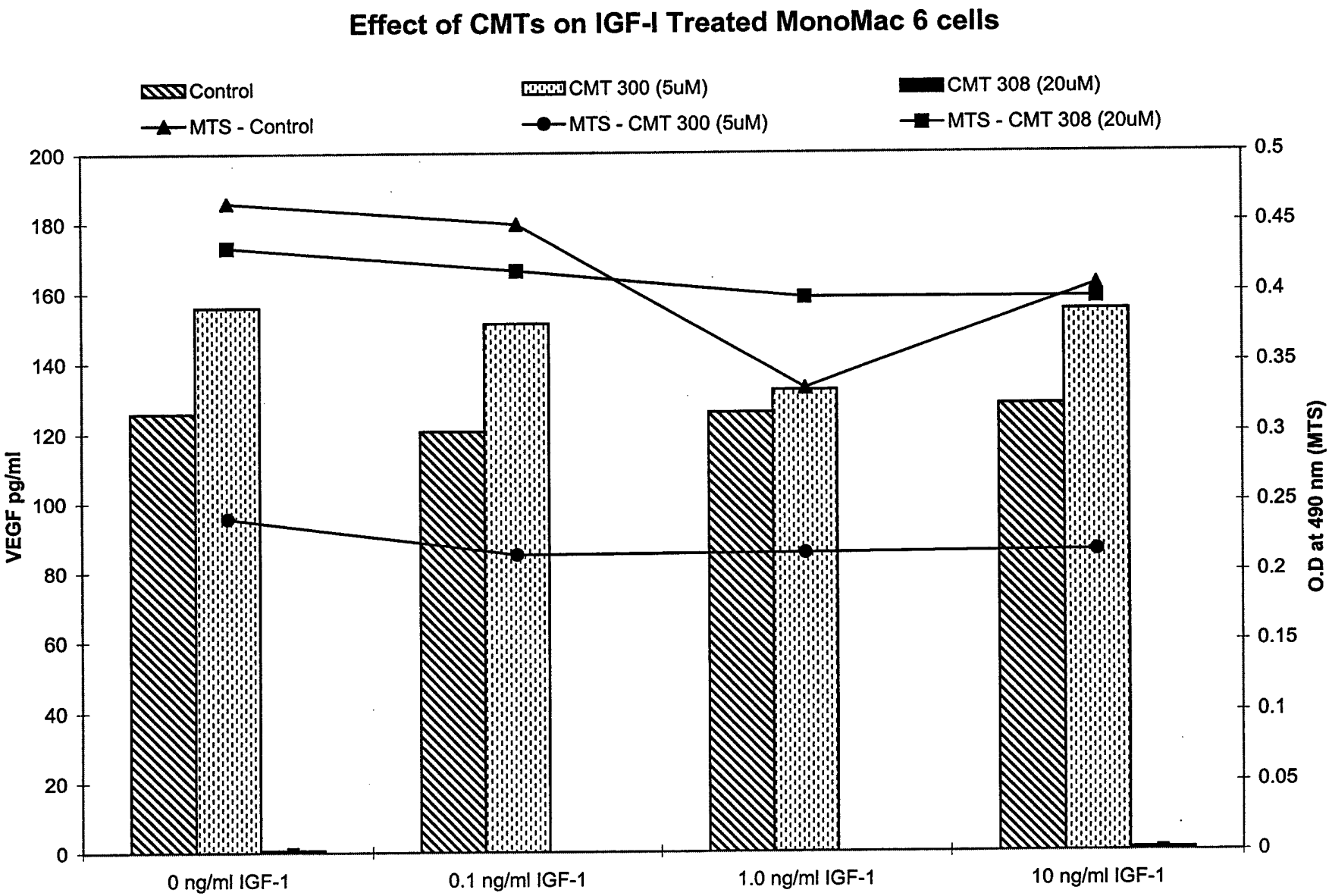

Figure 11
Page 1

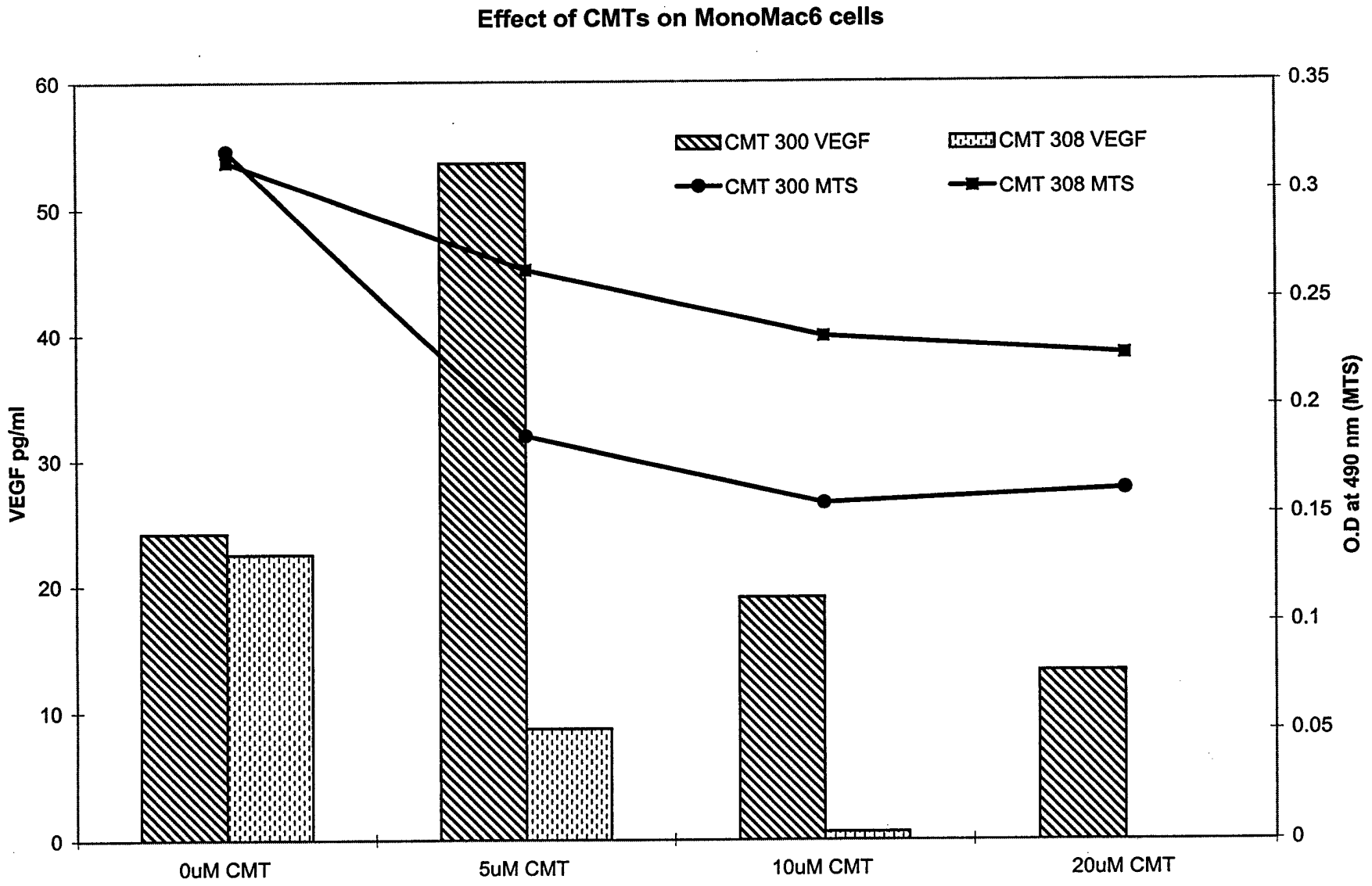

Figure 12

6/14/2002

ArmBCAnnRep02MonoMac6.xls Dose Response to CMTs

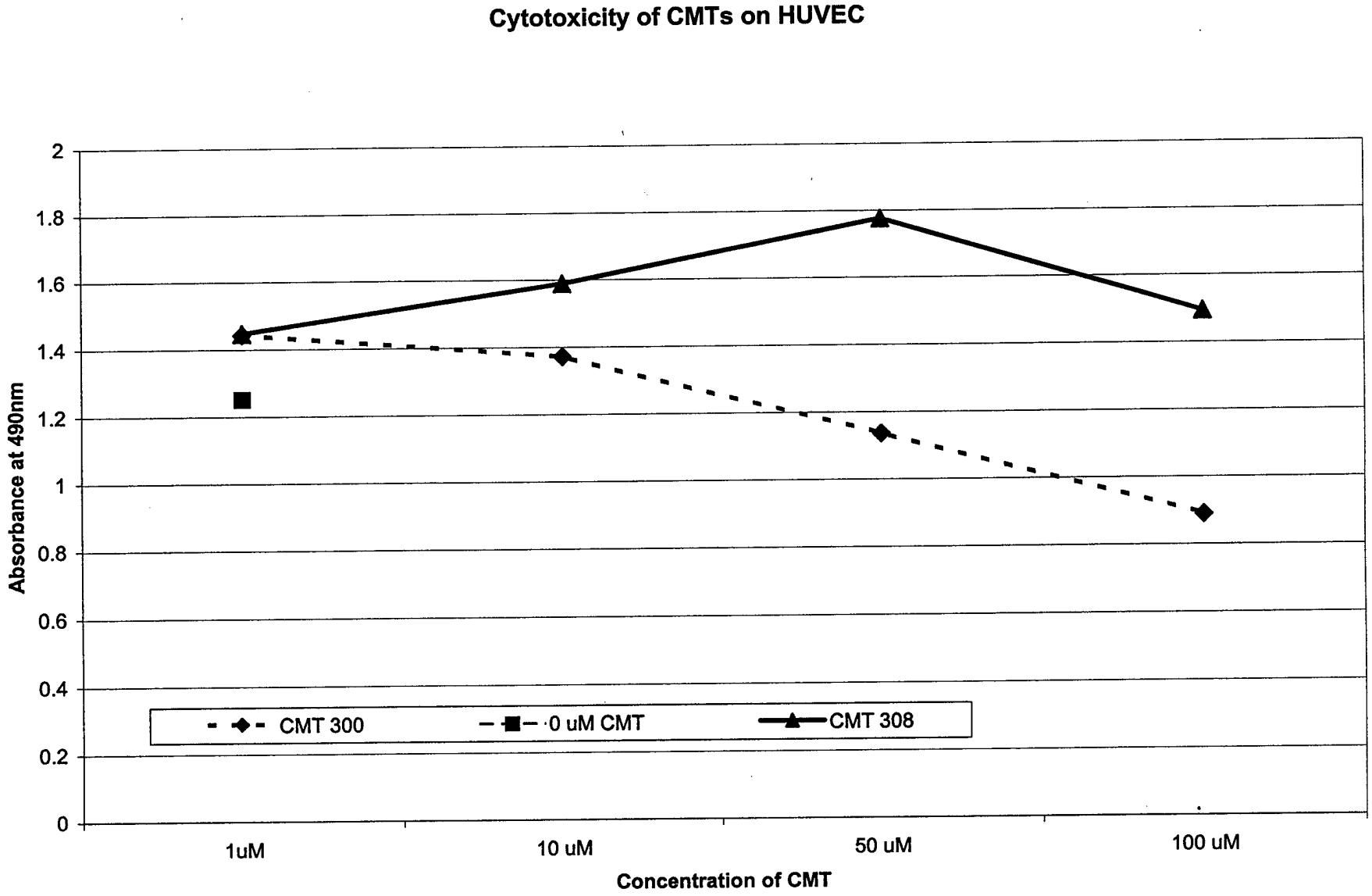
Cytotoxicity of CMTs on HUVEC
Absorbance at 490nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CMT 300
0 uM CMT
CMT 308
1uM
10 uM
50 uM
100 uM
Concentration of CMT

Figure 13

ANTIANGIOGENIC ACTION OF CHEMICALLY MODIFIED TETRACYCLINES IN BREAST CANCER

Sanford R. Simon and Mansi Kothari

Departments of Biochemistry and Pathology, SUNY at Stony Brook, NY 11794-8691

ssimon@notes.cc.sunysb.edu

Control of breast cancer may be achieved by a combination of interventions, including downregulation of the angiogenic response which maintains tumor growth and proliferation. Proangiogenic signals may eminate from the tumor cells themselves as well as from inflammatory cells which infiltrate the tumors. A nonantimicrobial chemically modified tetracycline, 6-deoxy-6-demethyl-4-de(dimethylamino)tetracycline (CMT-300) is currently being evaluated at the National Cancer Institute in Phase I trials on patients with a variety of solid tumors, and has been shown to reduce the angioproliferative response in Kaposi's Sarcoma. The 9-dimethylamino derivative of CMT-300 (CMT-308), induced less photosensitivity in vitro than the parent compound. Accordingly, we have studied the effects of CMT-300 and CMT-308 on two human breast tumor cell lines with different degrees of invasive and metastatic potential as well as a human monocytoid cell line which serves as a model of tumor-infiltrating macrophages. In the first year of research sponsored by this award, we have studied release of Vascular Endothelial Growth Factor (VEGF) by two breast tumor cell lines, MCF-7 (which retains estrogen responsiveness and is not highly invasive) and MDA-MB-231 (which is estrogen insensitive and is highly invasive), using an Enzyme-Linked Immunosorbant Assay (ELISA) for quantitation. Both lines release VEGF at levels which can be augmented in a dose-dependent fashion by Transforming Growth Factor-β (TGF-β). Consistent with the idea that MDA-MB-231 is a model of late stage aggressive breast cancer and MCF-7 is a model of earlier stage cancer, the former line releases higher levels of VEGF than the latter. The levels of VEGF from both lines are diminished when 20 μM CMT-308 (a noncytotoxic dose which is comparable to levels of CMT-300 reached in patients in the NCI Phase I trials) is present during culture. This diminution is especially marked in the presence of TGF-β, suggesting that CMT-308 may be affecting a signal transduction pathway which is activated in these tumor cell lines by TGF-β. Levels of VEGF released by both breast tumor lines are diminished much more by CMT-308 than by CMT-300. Because highly vascularized tumors are often also infiltrated with inflammatory cells, we examined the effects of the CMTs on VEGF production by a highly differentiated monocytoid cell line, Mono Mac 6. This cell line releases high levels of VEGF which are not affected by TGF-β, but 20 μM CMT-308 effectively abrogates all VEGF release. The results on VEGF release indicate that the CMTs may have utility in management of breast cancer by diminishing the pro-angiogenic signals released by the tumors and by infiltrating mononuclear cells. Because the CMTs have been found to be safe and well tolerated in cancer patients as well as normal volunteers, they have promise for rapid development as components of comprehensive therapeutic strategies for breast cancer management.

Supported by the U.S. Army- Medical Research and Materiel Command under DAMD17-01-1-0346.